Title of the Invention

Antisense oligonucleotide inhibiting IL-10 protein expression

Technical Field

The present invention relates to an antisense oligonucleotide hybridizing specifically with chromosomal DNA and/or mRNA encoding IL-10 (human interleukin-10) protein to thereby inhibit the IL-10 protein expression, and a pharmaceutical agent containing it as an effective component for the treatment of diseases such as atopic dermatitis .

Background of the Invention

Atopic dermatitis is a disease which repeats exacerbation and remission and has amyctic eczema as main lesion. The morbidity rate of it is considered to be 3 to 10% of the all population (Hiroshi Ueda: Hifuka MOOK atopic dermatitis, Kanehara Shuppan Co.,Ltd.12-18,1985). The mechanism of crisis of the atopic dermatitis has not been shown yet. It is, however, said that not only allergic reaction by way of IgE but also a factor of easy irritability of skin are largely concerned in the pathologic formation.

In the lesion of atopic dermatitis, macrophage or degranulation of eosinophil, cellular infiltration mainly of CD4 positive T cell are admitted. Allergen invaded in the skin crosslinks IgE on the mast cell, and liberates a neurotransmitter and cytokine, and enhances the rise of

vascular transmission or leukocyte migration. Further, allergen is trapped by Langerhans' cell and the like, and is presented as an antibody to T cells. The activated T cells produce a variety of cytokines and promote inflammatory (Cooper,K.D., J.Invest.Dermatol.,102,128,1994).

The treatments of atopic dermatitis are by means of a steroid or a non-steroid drug, and the steroid is now a principal pharmacotherapy. The therapy usually takes a long time, and the side-effects of the drug become a large problem. When the steroid is administered orally, suppression of adrenal function, suppression of bone mallow, opportunistic infectious diseases and the like which are systemic side-effects, must be considered. In addition, as the side-effects of steroid for external use, the teroid skin such as angiotelectasis, steroid flush or dermatrophy, the dermatomycosis such as candida or ringworm, the suppurative dermatitis such as carbuncle, and the virus diseases such as herpes simplex, may be induced and exacerbated. Further, the gradual decrease of administration must be carried out carefully about rebound. In view of the side-effect problems, a development of a new therapy in place of the present therapy, is desired strongly.

On the other hand, the detail mechanisms of crisis of atopic dermatitis have been shown clearly by the recent researches. Ohmen et al., have reported that in the inflammatory tissue of atopic dermatitis patient, interleuki-10 (IL-10) protein being cytokine is expressed

excessively and it is concerned in atopic dermatitis, that the IL-10 protein is secreted from macrophage cells, and that the treatment of atopic dermatitis will be possible if the excessive expression of IL-10 protein can be inhibited (J.D.Ohmen,et al.,J.Immunol.,154,1956, 1995).

The IL-10 protein was produced from Th2 cells by Fiorentino, 1989. It is a cytokine identified as a factor inhibiting cytokine production from Th1 cell (Fiorentino D.,et al.,J.Exp.Me.,170,2081,1989). Then, it was shown that the IL-10 protein was produced from Th2 cell, CD5 positive B cell, macrophage, keratinocyte, mast cell in the case of mouse, and that it was produced from a variety of cells such as Th0 cell, Th2 cell, activated T cell, monocyte, macrophage, activated B cell and the like in the case of human (Ishida H.,Jpn.J.Clin.Pathol.,42,843,1994).

As the synthesis of the mechanism of crisis of atopic dermatitis is proceeded, an attempt to apply the results of the synthesis to the treatment of diseases has been made.

The contents of it are to try to inhibit the IL-10 protein production which is regarded as a cause of the diseases and to improve the symptoms of diseases by inhibiting the function of the IL-10 protein, namely, a discussion on the therapy with an anti IL-10 protein neutralizing antibody is made (Ishida H., et al,J.Exp.Med.,179,305,1994).

In WO97/31532, the IL-10 antisense techniques for the purpose of treating AIDS related B cell lymphoma or chronic lymphocytic leukemia are disclosed. However, the inhibition

effects of the antisense oligonucleotide to +315 to +342 in the mRNA sequence of the human IL-10 protein to autocrine function of the IL-10 protein produced excessively from the B cell of the above patient, is only disclosed, and the one to another sequence is not disclosed at all.

Under such circumstances, a drug which has effects on atopic dermatitis and has little side-effect is desired, In particular, since the atopic dermatitis is seems to be caused from the IL-10 protein produced excessively from monocyte or macrophage, a development of a novel anti IL-10 preparation for atopic dermatitis treatment is desired.

Thus, the object of the present invention is to provide an antisense oligonucleotide which inhibits the IL-10 protein expression and can treat the diseases caused from the IL-10 protein such as atopic dermatitis, allergodermia, systemic lupus erythematosus (SLE), Epstein-Barr (EB) virus infectious diseases or lymphoma, and a pharmaceutical agent for treatment containing the antisense oligonucleotide or a derivative as an active component.

Disclosure of the Invention

The present inventors searched in order to obtain an effective therapeutic agent for intractable diseases caused by the IL-10 protein produced by monocyte or macrophage, and as the results, found antisense oligonucleotide sequences which inhibit strongly the IL-10 proteins production from monocyte or macrophage, and completed the present invention.

The antisense oligonucletide sequences are quite different sequences from the antisense oligonucleotide to +315 to +342 of the mRNA sequence disclosed in the above-described WO97/31532.

Namely, the present invention comprises an antisense oligonucleotide characterized in that it hybridizes specifically with the chromosomal DNA and/or RNA encoding human interleukin-10 (IL-10) protein to thereby inhibit the IL-10 protein expression, and it has any one or more of the following sequences (A) to (G):

(A) the sequence described in the SEQ ID NO:1 in the SEQUENCE LISTING,

(B) the sequence described in the SEQ ID NO:2 in the SEQUENCE LISTING,

(C) the sequence described in the SEQ ID NO:3 in the SEQUENCE LISTING,

(D) the sequence described in the SEQ ID NO:4 in the SEQUENCE LISTING,

(E) the sequence described in the SEQ ID NO:5 in the SEQUENCE LISTING,

(F) the sequence described in the SEQ ID NO:6 in the SEQUENCE LISTING,

(G) the sequence described in the SEQ ID NO:7 in the SEQUENCE LISTING,

and comprises a pharmaceutical agent containing the antisense oligonucletide or a derivative thereof as an effective component for the treatment of atopic dermatitis,

allergodermia, SLE, EB virus infectious diseases or lymphoma.

The present invention will be explained in detail, hereinafter.

The antisense oligonucleotide of the present invention contains one or more of the sequences of the SEQ ID NOS:1 to 7.

The antisense oligonucleotide described in SEQ ID NO:1 is a complementary base sequence to from +176 to +193 of human IL-10 mRNA,

The antisense oligonucleotide described in SEQ ID NO:2 is a complementary base sequence to from +181 to +198 of human IL-10 mRNA,

The antisense oligonucleotide described in SEQ ID NO:3 is a complementary base sequences to from +367 to +384 of human IL-10 mRNA,

The antisense oligonucleotide described in SEQ ID NO:4 is a complementary base sequences to from +637 to +654 of human IL-10 mRNA,

The antisense oligonucleotide described in SEQ ID NO:5 is a complementary base sequences to from +915 to +932 of human IL-10 mRNA,

The antisense oligonucleotide described in SEQ ID NO:6 is a complementary base sequences to from +1246 to +1263 of human IL-10 mRNA,

The antisense oligonucleotide described in SEQ ID NO:7 is a complementary base sequences to from +1249 to +1266 of human IL-10 mRNA.

The antisense oligonucleotide of the present invention may contain only one or more of the base sequences described in the SEQ ID NOS:1 to 7.

The antisense oligonucleotide of the present invention does not cause damage to macrophage and inhibits effectively the expression of IL-10 produced by macrophage. Among the SEQ ID NOS: 1 to 7 of the present invention, the SEQ ID NOS: 1,3,4,5 and 6 are excellent, and SEQ ID NOS: 3 and 4 are most excellent in the inhibition effects.

Even if the antisense oligonucleotides described in the SEQ ID NOS: 1 to 7 are used as antisense oligonucleotides in which one or more of the bases of the 5' side and/or 3'side are shortened, the activity of inhibiting IL-10 protein production is not disappeared, but in order to maintain the specificity to human IL-10 mRNA and not to have an influence on another genes, the antisense oligonucleotides described in SEQ ID NOS: 1 to 7 are preferably used in a minimum unit.

Further, although the activity of inhibiting IL-10 protein production is not disappeared even if the antisense oligonucleotides described in the SEQ ID NOS: 1 to 7 are used as the antisense oligonucleotides in which one or more of the bases of 5' side and/or 3'side are lengthened, when synthesizing the antisense oligonucleotide chemically, the longer the strand of nucleotide the higher the synthesizing cost. Thus these are preferably used in the strand length of the antisense oligonucleotides described in the SEQ ID NOS: 1 to 7.

The method of synthesizing the antisense oligonucleotide of the present invention is not particularly limited, for example, the phosphoramidite method using a conventional oligonucleotide synthesizer, the phosphorothioate method, phosphotriester type and the like may be used.

Further, in order to improve the stability and affinity to cells of the antisense oligonucleotide of the present invention, a derivative in which phosphoric ester group or hydroxyl group of ribose part may be substituted with another stable group may be used in the extent that the activity is not decreased conspicuously. The examples of the derivatives of the antisense oligonucleotide include, the one in which phosphoric ester group is substituted with thiophorphoric ester group, methylphosphonate group and the like, the one in which hydroxyl group of ribose part is substituted with alkoxy group such as methoxy and allyloxy, amino group or fluorine atom and the like. Among them, the one in which phosphoric ester group is substituted with thiophosphoric ester group is preferable in view of the inhibition effects of IL-10 protein production.

Even if the antisense oligonucleotide of the present invention is DNA type or RNA type, the inhibition effects on the IL-10 protein production may be expected, but the DNA type is more preferable since it has higher stability when administered in a living body.

The antisense oligonucleotide of the present invention

has a complementary and specific sequence to mRNA encoding human IL-10 protein, and can inhibit the functions of mRNA or DNA, i.e., any of translation to protein, transport into cytoplasm, or another activities necessary for general biological functions. Further, the antisense oligonucleotide of the present invention has no severe side-effect as shown in using steroids, and thus it can be used for the treatment of diseases safely and efficiently.

Since the antisense oligonuleotide of the present invention inhibits the IL-10 expression of monocyte or macrophage, it can effectively treat the diseases one of the reasons of which is considered to be the excessive expression of the IL-10 protein of monocyte and macrophage, for example, atopic dermatitis, allergodermia, SLE, EB virus infectious diseases, lymphoma and the like.

The antisense oligonucleotide of the present invention can be administered alone, but it can be administered as a pharmaceutical preparation by mixing with a pharmaceutical acceptable material.

For examples, when it is used as an injection, it can be prepared by dissolving the antisense oligonucleotide of the present invention into water, isotonic sodium chloride solution or dextrose solution and the like, and it may contain a buffer solution, a preservative, or a stabilizing agent and the like, depending on the necessity.

When it is used as an ointment, it can be prepared by dissolving or dispersing the antisense oligonucleotide of the

present invention into fat, emulsive or water soluble base, and it may contain a stabilizing agent, a pH controlling agent, a plasticizer, an emulsifying agent, a surface-active agent, a solubilizing agent, a wetting agent, a preservative, an antiseptic agent, a solvent or an absorption enhancing agent and the like, depending on the necessity.

When it is used as a creme agent, it can be prepared by dissolving or dispersing the antisense oligonucleotide of the present invention in an aqueous phase and emulsifying with an oil phase component such as a hydrocarbon, a higher alcohol and the like, and it may contain a stabilizer, a pH controlling agent, a plasticizer, an emulsifying agent, a surface-active agent, a solubilizing agent, a wetting agent, a preservative, an antiseptic agent, a solvent or an absorption enhancing agent and the like, depending on the necessity.

When it is used as a lotion or liniment agent, it can be prepared by dissolving or dispersing the antisense oligonucleotide of the present invention in a solvent, and it may contain a preservative, a pH controlling agent, an emulsifying agent, a suspending agent, a surface-active agent, a solubilizing agent, a wetting agent, a preservative, an antiseptic agent or an absorption enhancing agent and the like, depending on the necessity.

When it is used as a preparation for iontophoresis for percutaneous or permucosal administration, it can be prepared by adding the antisense oligonucleotide of the present invention in a conducting gel or a drug storage tank in a device,

and it may contain a stabilizer, a pH controlling agent, a surface-active agent, a solubilizing agent, a wetting agent, a preservative, an antiseptive agent, an absorption enhancing agent and the like, depending on the necessity.

These pharmaceutical preparation may contain another pharmaceutically acceptable and therapeutically useful component.

Further, if more efficient administration method is desired, it can be administered in combination with a pharmaceutical acceptable carrier.

As a carrier, for examples, a carrier containing lipid, such as liposome, fat emulsion and micelle as a main component, a peptide carrier such as Poly-lysine and polyornitine, a synthetic high molecular carrier such as polyethylene imine and polylactic acid/glycolic acid copolymer may be exemplified. Among them, a carrier having a cationic charge is preferable. In addition to it, the above-described carrier which is modified in order to improve the acceleration of the intake into cells and the tropic to the objected cells, may be used.

A plasmid or virus vector which is designed for expression of the anticense oligonucleotide of the present invention, is useful as a vector for gene therapy.

The administration rate of the antisense oligonucleotide of the present invention or an intimate mixture of the antisense oligonucleotide and a carrier, is not limited, such as oral administration, intravenous

administration, percutaneous administration, topical administration, intra-abdominal administration. However, the choice of an administration method having a higher effectivity to each disease, is preferable. For example, when it is used for therapy of atopic dermatitis, a percutaneous administration such as an administration using iontophoresis and an external preparation such as cream or ointment is preferable. When it is used for the therapy of SLE, intravenous administration is preferable. The dose is preferably increased or decreased depending on the degree of symptom of each disease and on the administration route, but it is 1mg/kg body weight to 1g/kg body weight, preferably 5mg/kg body weight to 500mg/kg body weight, more preferably 10mg/kg body weight to 300mg/kg body weight in the case of intravenous administration, as a standard.

Brief Description of the Drawings

FIG. 1 is a graph showing the effects on inhibition of IL-10 protein expression of the antisense oligonucleotide of the present invention.

Description of the Preferred Embodiments

Examples

The present invention will be described in more detail hereinafter by way of Examples. However, these Examples are shown only to assist the understanding of the present invention, and not to limit the scope of the invention.

In Examples, the SEQ ID NO:9 shows the cDNA base sequence of the human IL-10 protein (DNA Data Bank of Japan: DDBJ, Accession No.M57627).

In addition, the sequences according to the SEQ ID NOS:1 to 7 are antisense oligonucletide strands of the human IL-10 genes. The SEQ ID NO:1 corresponds to from +176 to +193 of the SEQ ID NO:9, the SEQ ID NO:2 corresponds to from +181 to +198 of the SEQ ID NO:9, the SEQ ID NO:3 corresponds to from +367 to +384 of the SEQ ID NO:9, the SEQ ID NO:4 corresponds to from +637 to +654 of the SEQ ID NO:9, the SEQ ID NO:5 corresponds to from +915 to +932 of the SEQ ID NO:9, the SEQ ID NO:6 corresponds to from +1246 to +1263 of the SEQ ID NO:9 and the SEQ ID NO:7 corresponds to from +1249 to +1266 of the SEQ ID NO:9, respectively.

Further, the SEQ ID NO:8 is a sequence of the antisense oligonucleotide strand of the mouse IL-10 protein gene, and is a negative control group having six mismatch sequences within from +1370 to +1387 of the SEQ ID NO:9 (Comparative Example 1).

In addition, the antisense oligonucleotides used in Examples are all phosphorothioate type, and those synthesized upon commission by Pharmacia Biotech Co. were used.

(Example 1)

Synthesis and Purification of Antisense Oligonucleotide

As to the antisense oligonucleotides according to the SEQ ID NOS:1 to 8 of the SEQUENCE LISTING were synthesized using a DNA synthesizer, Oligo Pilot II (manufactured by

Pharmacia Biotech Co.,Ltd.). Synthesizing based on the phosphorothioate method, these were purified by using FPLC director system (manufactured by Pharmacia Biotech Co.,Ltd.) based on the ion-exchange FPLC method.

(Test Example 1: Test of Effects on Inhibition of Human IL-10 Protein Expression)

(A) Transfer of the antisense oligonucleotide into culture cells

The inhibition effects on the human IL-10 proteins expression of the antisense oligonucleotides of the SEQ ID NOS:1 to 8, were examined in culture cell lines.

As the cells, a human monocytic macrophage, U937 cells (manufactured by Dainihon Seiyaku Co.,Ltd.) were kept under the conditions of 37°C, 5%CO_2, in a RPMI-1640 medium (manufactured by Kabushiki Kaisha Nikken seibutsu Igaku Kenkyusho) added by 10% fetal bovine serum (FCS: manufactured by Sanko Junyaku Co.,Ltd.) and an antibiotics (100unit/ml of penicillin (manufactured by GIBCO Co.,Ltd. and 100μg/ml of streptomycin (manufactured by GIBCO Co.,Ltd.)) (referred as FCS-containing medium, hereinafter). After seeding the U937 cells to have a concentration of 1×10^5/well in a 96 well plate, phorbol myristate acetate (PMA: manufactured by Wako Junyaku Co.,Ltd.) was added to have a concentration of 10ng/ml, and the cells were cultured for 12 hours (differentiation induction). After completing the culturing, the cells were washed with phosphate buffer solution (PBS), cultured in the FCS-containing medium for further 48 hours (treatment

interruption), washed with PBS, and each antisense oligonucleotide was added to have a concentration of 20 μM and cultured for four hours. After completing the culture, a solution of lipopolysaccharides (LPS: manufactured by Wako Junyaku Co.,Ltd.) and FCS-containing medium (the final concentration of LPS is 100 μg/ml) was added, and further cultured for 24 hours.

(B) Detection of the IL-10 protein by ELISA

Then, the human IL-10 protein in a supernatant collected from the medium solution obtained in the above (A), was determined by ELISA.

After adding 50 μl of anti-IL-10 monoclonal antibody (1 μg/ml 0.1M Na_2HPO_4 solution, pH9: manufactured by Pharmingen Co.,Ltd.) to 96 well plate (Sumitomo H-type) and leaving to stand it for all night at 4°C, the cells were washed with 200 μl of PBST (PBS containing (0.05% (v/v) Tween-20) four times. To avoid non-specific adsorption, after adding 200 μl of Blocking buffer (PBS containing 1% BSA) to each well and leaving to stand it at room temperature for 30 minutes, the cells were washed with 200 μl of PBST for three times. Then, the culture supernatant collected from the above (A) culture solution diluted with 100 μl of Blocking buffer/Tween (Blocking buffer containing 0.05%(v/v) Tween-20) and the IL-10 protein standard were added to the well, and left to stand for four hours at room temperature, these were washed with 200 μl of PBST four times. 100 μl of Biotin-labeled anti-IL-10 monoclonal antibody (manufactured by Pharmaingen

Co.,Ltd.) which was adjusted to have a concentration of 0.5 μg/ml with Blocking buffer/Tween solution, was added to the well, the cells were was left to stand for one hour at room temperature, and washed with 200μl of PBST six times. Further, 100μl of adipine peroxydase (manufactured by Pharmaingen Co.,Ltd.) which was adjusted to have a concentration of 0.5 μg/ml with Blocking buffer/Tween solution, was added to the well, and left to stand at room temperature for 30 minutes, and was washed with 200μl of PBST eight times. After adding 50μl of TMB (3,3',5,5'-tetramethy benzidine) substrate (manufactured by GIBCO Co.,Ltd.) to the well and leaving to stand for 30 minutes at room temperature, 50μl of 1M phosphoric acid was added as a reaction-stopping agent and the absorbency at 450nm was determined. The results were shown in Fig.1.

As it is clear from the results shown in Fig.1, it was proved that the effects on inhibition of the IL-10 protein expression by the antisense oligonucleotide (the SEQ ID NOS:1 to 7) of the present invention, are very excellent compared with the Non-Added Group and Comparative Example 1. Within the antisense oligonucleotides of the present invention, the SEQ ID NO:3>the SEQ ID NO:4>the SEQ ID NO:6>the SEQ ID NO:1>the SEQ ID NO:5>the SEQ ID NO:2>the SEQ ID NO:7 have larger effects on inhibition of IL-10 protein expression, in this order.

Any cytotoxicity was not observed by a microscopic examination.

Industrial Applicability

As described above, the antisense oligonucleotide of the present invention can inhibit effectively the IL-10 protein expression. Therefore, it is effective for intractable diseases caused by the IL-10 protein, for instance, atopic dermatitis, allergodermia, SLE, EB virus infectious disease, lymphoma and the like.